Soybean FGAM synthase promoters direct ectopic nematode feeding site activity

Zarir E. Vaghchhipawala, Jessica A. Schlueter, Randy C. Shoemaker, and Sally A. Mackenzie

Abstract: Soybean cyst nematode (SCN) resistance in soybean is a complex oligogenic trait. One of the most important nematode resistance genes, *rhg1*, has been mapped to a distal region of molecular linkage group G in soybean. A simplified genetic system to identify soybean genes with modified expression in response to SCN led to the identification of several genes within the nematode feeding sites. The genes were mapped to reveal their linkage relationship to known QTLs associated with soybean cyst nematode (SCN) resistance. One candidate, a phosphoribosylformylglycinamidine (FGAM) synthase (EC 6.3.5.3) gene, mapped to the same genomic interval as the major SCN resistance gene *rhg1* within linkage group G. Isolation of FGAM synthase from a soybean bacterial artificial chromosome (BAC) library revealed two highly homologous paralogs. The genes appeared to be well conserved between bacteria and humans. Promoter analysis of the two soybean homologs was carried out with the *Arabidopsis thaliana – Heterodera schachtii* system to investigate gene response to nematode feeding. The two promoters and their derived deletion constructions effected green fluorescent protein (GFP) expression within nematode feeding sites. The 1.0-kb promoter sequence immediately adjacent to the translation start site was sufficient to direct expression of GFP within syncytia. A wound-inducible element and a floral organ expression sequence were also identified within these promoters. Although a nematode-responsive element could not be identified, the observed expression of GFP within feeding sites supports the hypothesis that plant gene expression is redirected within feeding sites to benefit the parasite.

Key words: FGAM synthase, promoter analysis, syncytium, *Heterodera schachtii*, soybean cyst nematode.

Résumé : La résistance au nématode à kystes du soja (« soybean cyst nematode », SCN) est un caractère complexe et oligogénique. Un des gènes les plus importants, *rhg1*, a été cartographié dans la région distale du groupe de liaison G chez le soja. Un système expérimental simplifié a permis d'identifier plusieurs gènes du soja dont l'expression est modifiée en réponse à l'attaque par le SCN aux sites où le nématode se nourrit. Ces gènes ont été cartographiés pour déterminer leur relation avec des QTLs associés à la résistance au SCN. Un gène candidat, le gène codant pour la phosphoribosylformylglycinamidine (FGAM) synthétase (EC 6.3.5.3), est localisé dans le même intervalle génétique sur le groupe de liaison G que le gène majeur de résistance *rhg1*. Le clonage de ce gène au sein d'une banque de chromosomes bactériens artificiels (BAC) a révélé l'existence de deux paralogues très semblables. Ces gènes semblent avoir été très bien conservés des bactéries jusqu'à l'humain. Une analyse des promoteurs des deux homologues du soja a été réalisée à l'aide du système *Arabidopsis thaliana – Heterodera schachtii* pour étudier la réponse à l'attaque par un nématode. Les constructions contenant les deux promoteurs ou des délétions de ceux-ci ont induit l'expression de la protéine verte fluorescente (GFP) à l'endroit des sites où le nématode se nourrit. La région de 1,0 kb immédiatement en amont du site d'initiation de la traduction était suffisante pour induire l'expression génique au sein des syncitiums. Un élément cis de réponse aux blessures et un autre déterminant l'expression florale ont été identifiés au sein de ces promoteurs. Bien qu'un élément cis de réponse à l'attaque de nématodes n'ait pu être identifié, l'expression de la GFP aux sites d'attaque supporte l'hypothèse voulant que l'expression génique soit redirigée au bénéfice du parasite au sein des sites où il se nourrit.

Mots clés : FGAM synthétase, analyse de promoteurs, syncitium, *Heterodera schachtii*, nématode à kyste du soja.

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Z.E. Vaghchhipawala.[1] Department of Agronomy, Purdue University, West Lafayette, IN 47907, U.S.A.
J.A. Schlueter. Department of Zoology and Genetics, Iowa State University, Ames, IA 50011, U.S.A.
R.C. Shoemaker. USDA–ARS, CICGR, Department of Agronomy, Iowa State University, Ames, IA 50011, U.S.A.
S.A. Mackenzie.[2] Plant Science Initiative, University of Nebraska, Lincoln, NE 68588-0660, U.S.A.

[1]Present address: Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583, U.S.A.
[2]Corresponding author (e-mail: smackenzie2@unl.edu).

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Introduction

The soybean cyst nematode (SCN) *Heterodera glycines* (*Hg*) Ichinohe is considered the most economically debilitating disease-causing pathogen to affect soybean cultivation (Noel 1992), causing losses of up to one billion dollars annually (Kim et al. 1997). Several *Hg* types of SCN (Niblack et al. 2002) exist in the field (Riggs and Schmitt 1988) and several soybean genes that confer resistance have been identified. The most important of these genes have been mapped to linkage groups G and A2 of the soybean genetic map (Webb et al. 1995; Concibido et al. 1996; Meksem et al. 2001).

Several approaches have been undertaken to characterize nematode-responsive gene expression patterns within feeding sites of the soybean root. Changes in mRNA abundance were studied by in vitro translation to proteins (Hammond-Kossack et al. 1989; Potenza et al. 1996; Oberschmidt et al. 1997). Subtractive hybridization of cDNA libraries prepared from nematode-infected and uninfected roots has yielded "infection-specific" clones. This approach has been used in tomato plants infected with root-knot nematodes (Van der Eycken et al. 1996) and in potatoes infected with cyst nematodes (Niebel et al. 1995). Likewise, several PCR-based libraries have been constructed to permit the cloning of "giant cell-specific" transcripts (Wilson et al. 1994; Bird and Wilson 1994). Use of the differential display technique has yielded several interesting candidate genes in the *Arabidopsis–Meloidogyne* interaction (Vercauteren et al. 2001) and the soybean–SCN interaction (Hermsmeier et al. 1998). Promoter–GUS (β-glucuronidase) fusion (Opperman et al. 1994) and promoter trap (Barthels et al. 1997; Puzio et al. 1998) approaches have also been implemented to identify nematode-responsive loci.

In a previous report (Vaghchhipawala et al. 2001), we showed that several genes were upregulated within the syncytium during colonization of the root by SCN. We determined the map locations of some of the soybean genes responsive to nematode infection by locating them on the public soybean map (Shoemaker et al. 1996*a*). A particularly interesting candidate was phosphoribosylformylglycinamidine ribonucleotide (FGAM) synthase. This gene mapped to the same 3.0-cM interval of linkage group G as the major soybean SCN resistance locus *Rhg1* (Mudge et al. 1997).

FGAM synthase was of interest to our study because of its coincident location within the genomic interval containing *Rhg1* and its up-regulated expression within the nematode feeding site. The enzyme FGAM synthase catalyzes the fifth step of the de novo purine biosynthetic pathway, affecting the ATP-dependent transfer of the glutamine amido group to the C-4 carbonyl of FGAR (5′-phosphoribosyl-*N*-formylglycinamide). To investigate this soybean gene further, we isolated and characterized two FGAM synthase loci. The two loci were highly similar in sequence. Consequently, we wished to determine if the two copies had distinct functions and (or) expression profiles during development and syncytium formation.

Materials and methods

Vectors and strains

The genomic copies of FGAM synthase were isolated from a bacterial artificial chromosome (BAC library prepared from the partial *Hin*dIII digestion of genomic DNA of *Glycine max* L. Merr. 'Williams 82' (Marek and Shoemaker 1997). Gene promoter constructions used the vector pCAMBIA 1303 (http://www.cambia.org). Transgene constructions were introduced into ElectroMax™ DH10B cells (Life Technologies, Carlsbad, Calif.) of *Escherichia coli* via electroporation.

DNA gel blot analysis, PCR, and DNA sequencing procedures

DNA gel blot analysis was carried out using standard procedures (Sambrook et al. 1989). DNA sequencing was accomplished using the fluorescently labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Intl., Buckinghamshire, England) in an ALFexpress automated sequencer (Pharmacia, Biotech AB, Umeå, Sweden). The polymerase chain reaction (PCR) was carried out using genomic DNA from transgenic *Arabidopsis* leaves prepared according to published protocol (Li and Chory 1998) as template. Primers were designed from the *uidA* sequence to amplify a product of approximately 1189 bp.

Genomic, plasmid, and BAC DNA preparations and sequence homology searches

Genomic DNA was prepared by the method of Vallejos et al. (1992). Plasmid DNA preparations were carried out using the CONCERT™ plasmid miniprep kit (Life Technologies), whereas BAC DNA was prepared using a modified alkaline lysis protocol (Felicielo and Chinali 1993). The "Bestfit" function of GCG package software "SEQWEB" was used to identify sequence homologies, and the "motifs" function was used to locate protein motifs of interest.

Preparation of promoter constructions

The subcloning of the promoter region was carried out in the vector pCAMBIA1303, which incorporates the reporter genes β-glucuronidase (GUS) and enhanced green fluorescent protein (GFP) under the control of the CaMV 35S promoter. Cloning was accomplished by excising the 35S promoter from the vector by digestion with enzymes *Bam*HI and *Nco*I, and introducing putative promoter fragments from the two identified FGAM synthase genes, *FGAM1* and *FGAM2*. Promoter inserts (2.48 kb) and their derived truncations were generated by PCR amplification with primers designed to contain *Bam*HI and *Nco*I restriction sites.

Generation of *Arabidopsis* transformants

The transformation of *Arabidopsis thaliana*, grown in a 16 h light : 8 h dark regimen, was carried out using the floral dip method (Clough and Bent 1998). *Agrobacterium tumefaciens* C58C1 (provided by Dr. Thomas Clemente, University of Nebraska, Lincoln, Nebr.) was used to transform *Arabidopsis* ecotype Columbia. Transgene constructions were mobilized into the *Agrobacterium* strain via electroporation. Upon transformation, selection of transgenic plants was carried out by plating surface-sterilized seeds on 0.5× MS-B medium with 2% w/v sucrose, vitamins, and 20 mg hygromycin/L. Selected plants were subjected to GUS staining (Jefferson et al. 1987), PCR analysis, and DNA gel-blot analysis before inclusion in the nematode assay.

GUS staining and microscopy procedures

Plant tissues were immersed in X-gluc (0.8 mg/mL) solution and kept overnight at 37 °C for color development. After staining, 70% v/v ethanol was added for clearing of pigments, following the procedure of Jefferson et al. (1987). The infection of transgenic *Arabidopsis* roots by *Heterodera schachtii* was examined for GFP fluorescence with a confocal laser scanning microscope (CLSM) (Bio-Rad, Hercules, Calif.).

Heterodera schachtii infection assays of *Arabidopsis* transformants

Seeds from confirmed transgenic *Arabidopsis* plants were germinated on selective media as described above, then transferred to individual wells of a 12-well petri plate containing 1.5 mL of a modified Knop's medium (Sijmons et al. 1991), minus antibiotics. Infection was carried out on 11- to 13-day-old seedlings whose roots had penetrated into the medium. Each 12-well plate contained 10 individual T_1 transgenic seedlings derived from one independent transformant; the last two plants in the plate served as uninoculated controls. This system, following the procedure of Baum et al. (2000), provided ample experimental replications without undue contamination. The plants were inoculated near the roots with 50–100 surface-sterilized J2 juveniles of *Heterodera schachtii* suspended in 1.5% w/v low-melting-point (LMP) agarose. After 6–8 days incubation in a growth chamber at 25 °C and 16 h light : 8 h dark, allowing for feeding site establishment on the roots, plants were examined for GFP expression at root feeding sites by CLSM. Subsequently, GUS expression was assayed by filling the entire well with X-gluc staining solution and incubating at 37 °C overnight. Clearing of tissues involved adding 70% v/v ethanol, and cleared roots were observed under the dissecting microscope.

Surface sterilization of *Heterodera schachtii* J2 juveniles

Worms freshly hatched after a 2–3-day incubation in a hatch chamber in 3.14 mM $ZnSO_4$ were used for inoculation. Juveniles were counted in a haemocytometer and approximately 100 000 individuals were placed in a sterile 50-mL centrifuge tube. The samples were washed once in sterile distilled water by pelleting at 350–620 *g* for 3 min in a centrifuge using a swinging bucket rotor and no brake. The nematodes were resuspended in 50 mL of 0.001% hibitane (chlorhexidine, diacetate salt, Sigma No. C6143) for 30 min, mixing continuously. The sample was centrifuged at 350 *g* for 3 min and resuspended in 50 mL of 0.01% w/v $HgCl_2$. This suspension was incubated for 7 min, including the time to pellet the worms and remove supernatant. The sample was centrifuged to remove the $HgCl_2$, followed by three washes with sterile distilled water. After the last wash, enough 1.5% w/v LMP agarose was added to achieve the desired final concentration of nematodes, and the sample was maintained at 37 °C. The slurry was pipeted over roots in each well. J2 motility was observed after the LMP agarose had solidified.

Results

Assembly of soybean FGAM synthase gene contigs

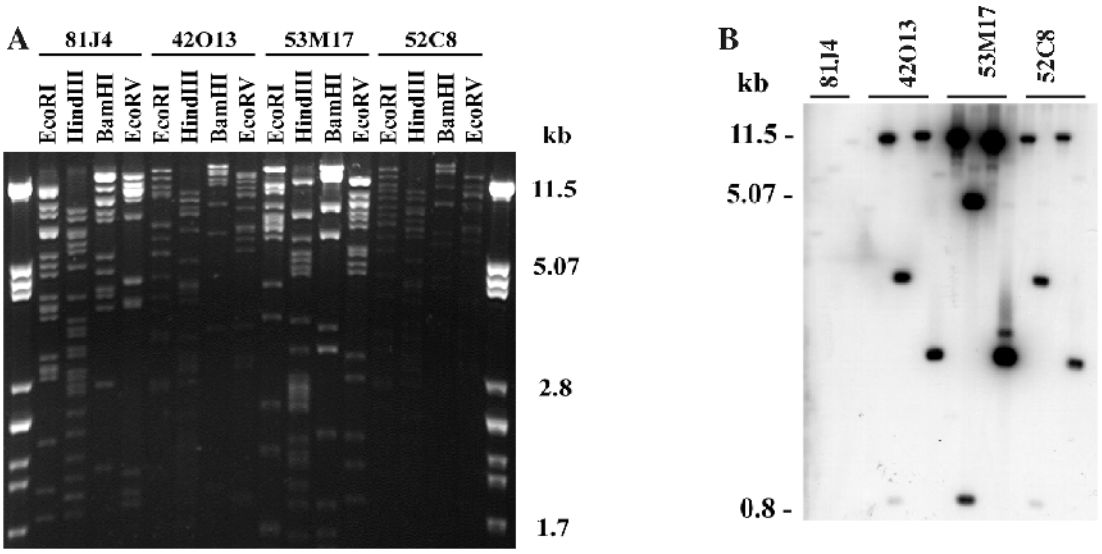
The sequence of FGAM synthase cDNA (AF000377) was used to generate two primers for use in RT-PCR. Primers 113 (5′-GCTATTGATGGAGGGAAAGACAG-3′) and 114 (5′-GCCATCTCTAAGGCACAAACTAG-3′) were used to screen soybean genomic BAC library DNA pools by PCR. The search yielded four putative hits and the corresponding BAC clones 81J4, 42O13, 53M17, and 52C8 were selected. The four BAC clones were digested with *Not*I enzyme and subjected to pulsed field gel electrophoresis to estimate insert sizes ranging from 110 to 160 kb (data not shown). To assemble the BAC clones into contigs, multi-enzyme DNA digestions were separated by agarose gel electrophoresis. Figure 1A shows that BAC clones 42O13 and 52C8 shared several bands in common, whereas the fingerprint of BAC 53M17 shared fewer bands. BAC 81J4 had a distinct banding pattern. Overlaps were confirmed by DNA gel blot hybridization (Fig. 1B). When probed with the FGAM synthase cDNA clone (890 bp), BACs 42O13 and 52C8 produced identical hybridization patterns, whereas the pattern produced by BAC 53M17 differed. A very faint hybridization signal was detected in BAC 81J4, suggesting that the FGAM homology contained within this locus was weak (Fig. 1B). The two distinct forms of FGAM synthase represented in BACs 53M17 and 42O13/52C8 were henceforth referred to as *FGAM1* and *FGAM2*, respectively. Digestion of 'Williams 82' genomic DNA with *Hha*I also revealed two prominent and one faint band, consistent with presence in the genome of two homologous loci and one divergent sequence (data not shown).

Genetic mapping of the original FGAM cDNA in the soybean genome indicated that at least one copy of the FGAM loci is derived from linkage group G at the same map location as the major SCN resistance gene, *Rhg*1. Mapping data were derived from a mapping population of 57 F_2 individuals and a recombinant inbred line (RIL) mapping population of 100 individuals (Vaghchhipawala et al. 2001). BAC analyses confirmed that the FGAM locus is duplicated. However, the location of the duplicate FGAM locus was not determined. Overlapping fragment analysis was used to determine full-length genomic sequence of genes *FGAM1* and *FGAM2* using the FGAM synthase cDNA clone to generate end probes (data not shown). At the 5′ end of each gene, approximately 2.5 kb of promoter sequence was also determined.

Characterization of the duplicate FGAM synthase loci

DNA sequence analysis of *FGAM1* (AY178840) and *FGAM2* (AY178839) revealed open reading frames of 3939 bp and 3940 bp, respectively. The two DNA sequences were 95.5% identical. Cluster analysis to assess amino acid sequence conservation among homologous FGAM synthase sequences available for soybean, *Drosophila*, human, and *E. coli* revealed highest sequence conservation among these genes within the ATP-binding domain and three glutamine-binding domains as shown in Fig. 2A. Dendrogram analysis of 12 FGAM sequences from GenBank revealed a separate clustering of microbial and higher eukaryotic sequences. Among the higher eukaryotic genes identified plant and animal sequences form distinct groups (data not shown). Sequence analysis of the 2.5-kb promoter region of the *FGAM1* and *FGAM2* genes revealed 85% identity. Scanning of the promoter sequences for various motifs revealed the presence of a stress-response element (STRE) (Schuller et

Fig. 1. Detection of FGAM synthase genes within four selected BAC clones. (A) Restriction endonuclease analysis of four BAC clones involved in multiple enzyme digestions to identify contig overlaps. A 5-μg sample of BAC DNA was digested with 15 U enzyme. Digestions were fractionated in 0.8% w/v agarose at 2.5 V/cm overnight. *Pst*I-digested λ DNA was used as a marker (M). (B) DNA gel-blot analysis of A using the 0.89-kbp FGAM cDNA clone as probe.



al. 1994) within the promoter of *FGAM1* (nt 2361–2369 from 5′ end) with 97% conservation. This element is shown to activate transcription of a yeast gene in response to a variety of stress stimuli (Schuller et al. 1994). Alignment of the two promoter sequences to the *wun1* wound-inducible promoter from potato, inducible during cyst nematode infection (Hansen et al. 1996), revealed a 39-bp interval with 95% sequence identity within the *FGAM1* promoter, but only 68% identity within the *FGAM2* promoter (Fig. 2B).

Promoter analysis in the *Arabidopsis thaliana* – *Heterodera schachtii* system

To determine which FGAM synthase gene was responsive to nematode infection, we conducted transgenic promoter analysis in the established *A. thaliana* – *H. schachtii* system (Sijmons et al. 1991). This system appears to parallel cellular events of the soybean–SCN infection process (Golinowski et al. 1996). To determine which promoter intervals were serving to modify gene expression within syncytia, we developed two deletion constructions from each full-length promoter. The deletions were made at the 5′ end of each original 2.48-kb promoter, leaving 1.5-kb and 1.0-kb sequences immediately 5′ to the translation start site in association with GUS (*uidA*) and *gfp* reporter genes as diagrammed in Fig. 3. The most divergent interval between the two promoters was located between nucleotides –1483 and –1983 (in relation to the +1 translation start site) in the *FGAM2* promoter and nucleotides –1314 and –1014 (in relation to +1 start site) in the *FGAM1* sequence. Within this region, there exists a stretch of sequence of 70 nucleotides in the *FGAM2* promoter that is absent from the *FGAM1* promoter. To test whether the divergent sequences might account for nematode responsiveness, two deletion constructions containing this region, Pr1-1.5 (*FGAM1*) and Pr2-1.5 (*FGAM2*), were derived. The effect of deleting these divergent regions was assessed with constructions Pr1-1.0 and Pr2-1.0 (Fig. 3).

FGAM1 and *FGAM2* promoter expression

Transformants for the six promoter constructions of *FGAM1* and *FGAM2*, as well as the vector control, were stained with X-gluc solution. Two independent vector-transformed control lines, harboring the 35S promoter fused to GUS–GFP, produced GUS staining in leaves, inflorescence, stem, and roots (Fig. 4A). Five independent transformants containing the full length (2.48-kbp) FGAM synthase promoter from gene *FGAM2* (Pr2-2.5) were evaluated for GUS expression, and none produced detectable GUS staining in any part of the seedling including inflorescence (Fig. 5A). The same results were obtained for the four independent transformants of deletion construction Pr2-1.5 (data not shown) and for seven transformants of construction Pr2-1.0 (data not shown).

Experiments with the 2.48-kb full-length *FGAM1* promoter (Pr1-2.5) produced four independent transformants. With some minor plant variation, Pr1-2.5 transformants showed GUS staining in leaf margins and veins, the root tip and lateral root meristems and inflorescence with the exception of anthers (Fig. 6A). The *FGAM1* deletion constructions, Pr1-1.5 (two events) and Pr1-1.0 (two events) showed no visible GUS staining anywhere in the seedling including flowers (data not shown). Non-transformed seedlings produced no GUS staining (Fig. 7A). These results imply that the two promoters differ markedly in strength as a consequence of sequences located more than 1.5 kb from the translation start site in *FGAM1*.

Promoter expression analysis in *H. schachtii* inoculated *Arabidopsis* roots

Twelve individual T_3 progeny per gene constructions were used in the *H. schachtii* infection assay carried out in 12-well plates. Two plants served as uninoculated controls. Each plant was infected with 50–100 J2 juveniles, maintained in the growth chamber for 6 days, and then observed under CLSM for GFP expression within feeding sites.

Fig. 2. (A) Sequence alignments of the soybean FGAM synthase gene with other known FGAM sequences. Multiple alignment of amino acid sequences (Higgins and Sharp 1988) for genes *FGAM1*, *FGAM2*, *Drosophila melanogaster*, *Homo sapiens*, and *Echerichia coli* using the ClustalW program. Only conserved domains are shown. Identical amino acids are red, similar amino acids are blue, and the remainder are black. The eight conserved amino acids of the ATP-binding domain (overlined) are shown in green, whereas the three glutamine-binding domains are shown in purple. (B) Bestfit analysis of the sequence homology of promoter regions of *FGAM1* and *FGAM2* genes with the *wun1* promoter from potato (Hansen et al. 1996).

A

ATP Binding

```
FGAM1        309 PYPGAETGAGGRIRDTHATGRGSFVQAATAGYCVGNLNTPGFYAPWEDPSFTYPSNLAPP
FGAM2        309 PYPGAETGAGGRIRDTHATGRGSFVQAATAGYCVGNLNTPGFYAPWEDSSFTYPSNLAPP
DM-FGAM      324 PFSGATTGTGGRLRDVQGVGRGGVPIAGTAGYCVGALHIPGYKQPYEPLDFKYPATFAPP
HUMAN-FGAM   352 PFSGATTGTGGRIRDVQCTGRGAHVVAGTAGYCFGNLHIPGYNLPWEDLSFQYPGNFARP
ECOLI-FGAM   304 PWPGAATGSGGEIRDEGATGRGAKPKAGLVGFSVSNLRIPGFEQPWEE-DFGKPERIVTA
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Gln Binding

```
FGAM1       1051 EEGSNGDREMAAAFYAAGFEPWDITMSDLLNGKISLLDFRGIVFVGGFSYADVLDSAKGW
FGAM2       1051 EEGSNGDREMAAAFYAAGFEPWDITMSDLLNGKISLQDFRGIVFVGGFSYADVLDSAKGW
DM-FGAM     1092 EEGVNSEREMMACLLRANFEVHDVTMSDLLQGTASVSQYRGLIFPGGFSYADTLGSAKGW
HUMAN-FGAM  1103 EEGSNGDREMADAFHLAGFEVWDVTMQDLCSGAIGLDTFRGVAFVGGFSYADVLGSAKGW
ECOLI-FGAM  1047 EQGVNSHVEMAAAFHRAGFDAIDVHMSDLLTGRTGLEDFHALVACGGFSYGDVLGAGEGW
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Gln Binding

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FGAM1       1111 SASIRFNESVLQQFQEFYKRPDTFSLGVCNGCQLMALLGWVPGPQVGGVHGAGG----DL
FGAM2       1111 SASIRFNESVLQQFQEFYKRPDTFSLGVCNGCQLMALLGWVPGPQVGGVHGAGG----DL
DM-FGAM     1152 AANILHNPRLLPQFEAFKRRQDVFSLGICNGCQLMTLIGFVGSAKSEVGADP--------
HUMAN-FGAM  1163 AAAVTFHPRAGAELRRFRKRPDTFSLGVCNGCQLLALLGWVGGDPNEDAAEMGPDSQPAR
ECOLI-FGAM  1107 AKSILFNDRVRDEFATFFHRPQTLALGVCNGCQMMSNLRELIPGSE----------L--W
```

Gln Binding

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FGAM1       1227 VHSELAPIRYCDDAGNPTEAYPFNANGSPLGVAAICSPDGRHLAMMPHPERCFLMWQFPW
FGAM2       1227 VHSELAPIRYCDDAGNPTEAYPFNVNGSPLGVAAICSPDGRHLAMMPHPERCFLMWQFPW
DM-FGAM     1263 QSEQLVTLQYVDDVGKPTELYPLNPNGSPQGIAGLCSSDGRHLALMPHPERCSSMYQWPY
HUMAN-FGAM  1283 EARGLAPLHWADDDGNPTEQYPLNPNGSPGGVAGICSCDGRHLAVMPHPERAVRPWQWAW
ECOLI-FGAM  1213 ESKGLVALRYVDNFGKVTETYPANPNGSPNGITAVTTESGRVTIMMPHPERVFRTVSNSW
```

B

FGAM1 promoter alignment to *wun1* promoter

Percent Similarity: 94.595 Percent Identity: 94.595

```
FGAM1       861 AACCGATATATATATATATATATATATATATATCAAT 897
                ||||||||||||||||||||||||||||||||||  ||
Wun1         69 aaccgatatatatatatatatatatatatatatat 105
```

FGAM2 promoter alignment to *wun1* promoter

Percent Similarity: 68.354 Percent Identity: 68.354

```
FGAM2      1332 ATATCGTGTAAATTTGTTTAAATTTATTCTATAAAAAATACTTATTTTAA 1381
                ||||||| |||| | || | || |  |  | | | |||  ||  ||||
Wun1        526 atatcgttaaaatattttaatatcttgttgaaatataatttttttatttag 575

           1382 TAAAATAA....ACAATTTTTTTTTCTTTTTTA 1410
                ||||||||    | ||||  ||||| || || |
            576 taaaataatatgagaattaatttttttttattaa 608
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Fig. 3. Diagrammatic representation of promoter organization for genes *FGAM1* (A) and *FGAM2* (B). Promoter deletions were generated from the –2500 bp end using PCR. Domains identified in these promoters via functional or sequence analysis are indicated. WR indicates a wound response element identified by sequence homology and shown functionally in Pr1-2.5 plants. STRE designates a stress-response element.

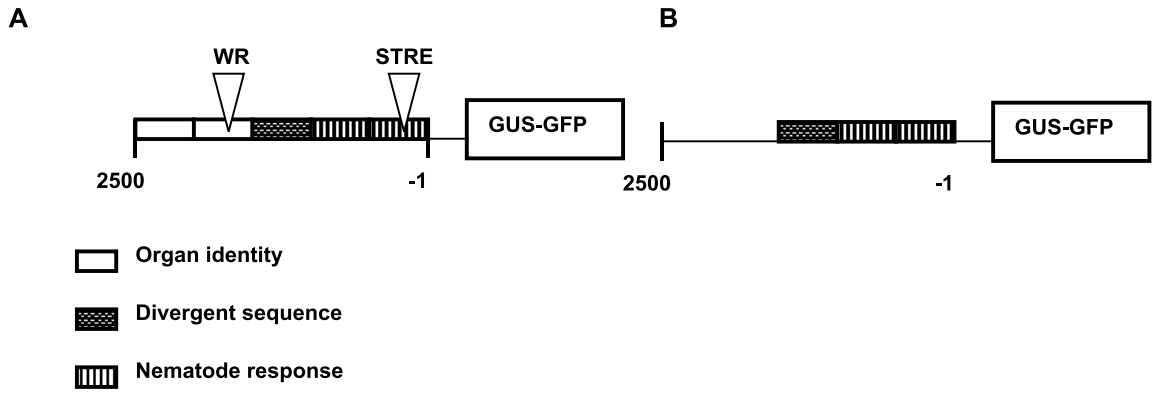
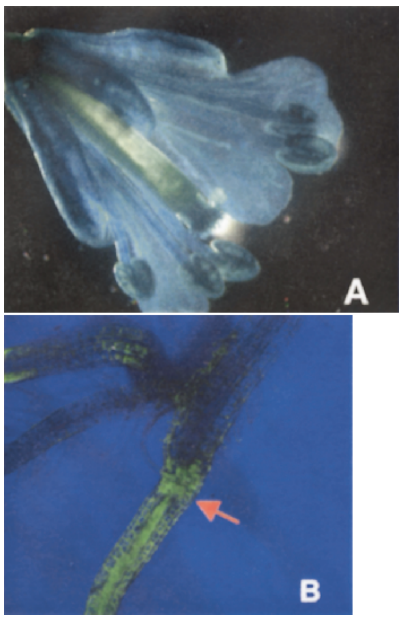
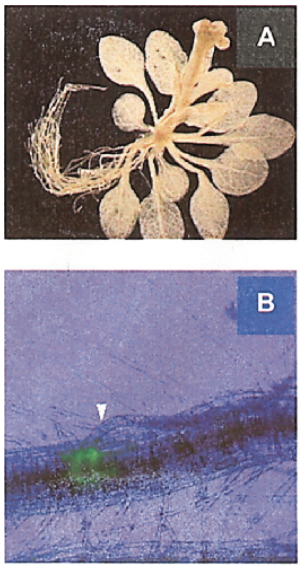


Fig. 4. Reporter gene expression in transformed *Arabidopsis* plants and nematode-challenged roots. (A) GUS expression directed by the CaMV 35S promoter within various organs of the seedling and roots. (B) Confocal microscopic examination of CaMV 35S::GFP expression at a nematode feeding site.



Roots of the vector control showed a uniform green fluorescence, and did not show significant elevation of GFP fluorescence at the sites of infection (Fig. 4B). Localized at the region of the root where a nematode had established a syncytium, a significant elevation of GFP expression above background was observed in all *FGAM1* and *FGAM2* promoter constructions (Figs. 5B and 6B). This observation was documented at least five times in each inoculated well (50 replicates for each independent transformant) for all promoter constructions. No localized elevation of GFP expression was seen in the uninoculated controls (data not shown). Instances in which the nematode had penetrated the root tissue, but had not yet established a feeding site, showed no localized elevation of GFP (Fig. 7B). This observation suggests that the establishment of a feeding site was necessary for the enhancement of local GFP expression levels, and implies that the elevated expression was not simply a localized wound response.

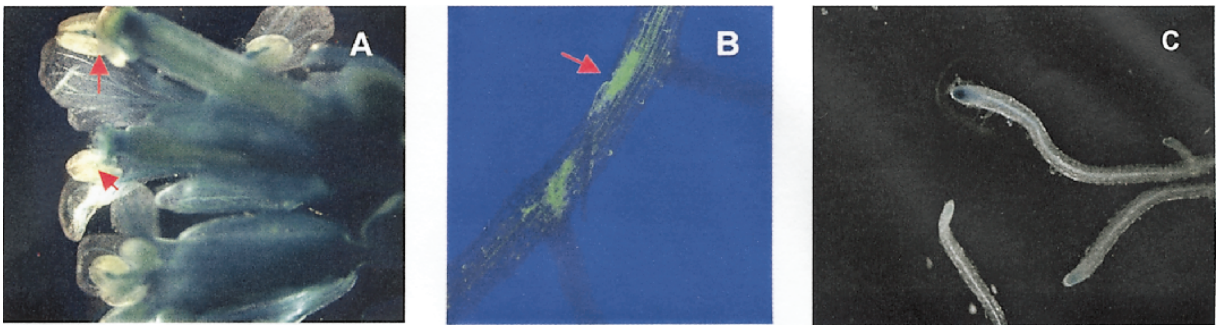
Fig. 5. (A) Reporter gene expression from constructions containing the *FGAM2* promoter (Pr2-2.5, Pr2-1.5, and Pr2-1.0) in *Arabidopsis* plants and nematode-challenged roots. Test for GUS expression within entire seedling of a Pr2-2.5-transformed plant. Identical results were obtained with all *FGAM2*-derived constructions (data not shown). (B) Localized elevation of GFP expression at nematode feeding site in the Pr2-2.5 transformant. The arrowhead indicates a nematode. Transgenic plants containing deletion constructions Pr2-1.5 and Pr2-1.0 produced similar results (data not shown).



Wound response

Sequence homology data indicated that the *FGAM1* gene promoter contains a 39-bp sequence with 95% sequence identity to the *wun1* wound inducible promoter from potato. The *FGAM2* gene promoter displayed only 68% sequence identity to the *wun1* promoter. A leaf from each transformant was excised from the seedling and assayed for GUS expression. Of all tranformants tested, one containing the full-length *FGAM1* promoter construction (Pr1-2.5)

Fig. 6. Reporter gene expression in FGAM1-derived transformants. (A) GUS staining of various plant organs in Pr1-2.5 transgenic plants with the notable exception of anthers (arrows). Transformants containing deletion constructions Pr1-1.5 and Pr1-1.0 produced no detectable GUS staining (data not shown). (B) Local elevation of root GFP expression within feeding sites of a Pr1-2.5 transformant. Similar results were obtained for the FGAM1 deletion constructions (data not shown). (C) GUS staining in root meristems of a Pr1-2.5 transformant. Staining of lateral meristems was also observed (data not shown).



showed what appeared to be a wound response. The excised leaf produced a visible staining pattern in the area around the wounded edge, while the remainder of the leaf remained unstained (Fig. 7C). This observation suggests that the *FGAM1* promoter causes a weak wound response. None of the transformants containing the *FGAM2* full length or deletion promoter constructions showed evidence of wound response (data not shown). These results, again, imply that the nematode responsive expression observed in all transformants did not represent a general wound response.

Discussion

This report describes the isolation and characterization of duplicate copies of the *FGAM* synthase gene from soybean. This gene was identified by differential display analysis and confirmed by RT-PCR to be up regulated within the feeding sites of *Heterodera glycines* in soybean roots (Vaghchhipawala et al. 2001). Isolation and characterization of the gene from 'Williams 82' revealed the presence of three copies of the gene, two with high sequence homology and one distantly related. The presence of multiple gene copies was anticipated given the duplicated nature of the soybean genome (Shoemaker et al. 1996*b*).

The *FGAM1* gene was encompassed within BAC 53M17, while *FGAM2* resides within the BAC 42O13/52C8 contig. The high sequence similarity between the genes suggest that the two loci have likely arisen by gene duplication. The degree of sequence identity between the two open reading frames (95.5%) and promoter regions (85%) implies that the duplication occurred fairly recently in evolutionary terms. Although the two gene copies show high protein sequence identity, an estimation of the coalescence time following the procedure of Lynch and Conery (2000) yields a date of approximately 11 million years ago. The two loci apparently continue to carry out duplicate functions in differing spatial and temporal patterns or in response to varying stimuli.

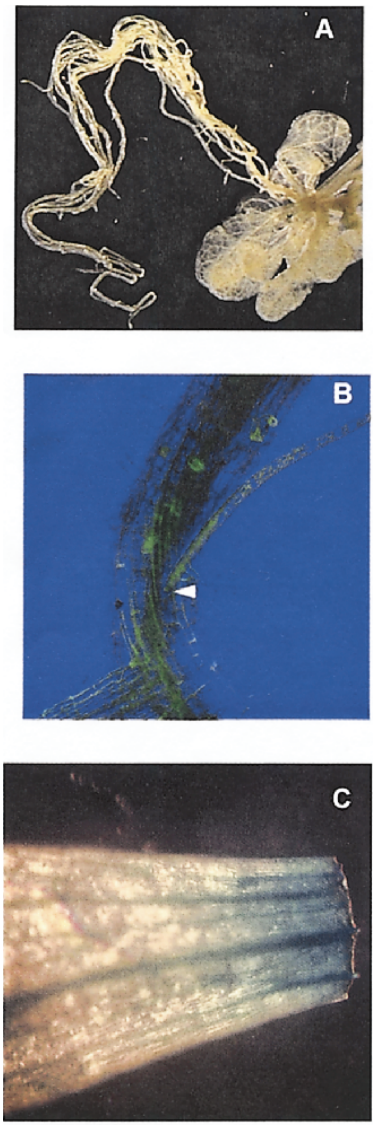
Evidence for multi-gene copies in soybean is extensive. A recent study (Jin et al. 1999) reported at least 12 classes of β-1,3-glucanase genes displaying divergent gene expression patterns. Members of a BURP domain-containing protein family, from soybean were also shown to possess diverse expression patterns (Granger et al. 2002). Mahalingam et al. (1999) identified two copies of a polygalacturonase gene, also from soybean, with expression up-regulated during syncytium establishment. Yamamoto and Knap (2001) identified three soybean orthologs of *A. thaliana* receptor-like protein kinases showing high sequence homology and predicted to have arisen from recent duplication events. The advantage of gene redundancy in soybean and other plant genomes is not known, but it has been suggested that though members of a gene family generally retain a set of standard functions, they acquire unique expression patterns and responses to environmental stimuli. It has been proposed that tissue specificity is an early step in functional divergence of a gene family, while divergence at the amino acid level occurs later (Pickett and Meeks-Wagner 1995). The differential expression of *FGAM1* and *FGAM2* and the observed divergence between their promoters are consistent with this hypothesis.

The essential function provided by FGAM synthase would predict its activity in areas of rapid cell proliferation. These tissues should include reproductive organs and apical and lateral meristems. This anticipated pattern of expression was evident in the GUS expression assays for *FGAM1* full-length promoter (Pr1-2.5) (Fig. 6C). A surprising exception was the pollen sacs, in which no *FGAM1* expression was detected. Possibly, sequences for anther expression were present further upstream to the region tested and were omitted from our constructions, or a different FGAM synthase copy might be expressing within anther tissues. Lack of detectable GUS expression in the *FGAM1* promoter deletions (Pr1-1.5 and Pr1-1.0) suggests that enhanced expression levels or tissue specificity of expression may reside within the interval 1.5 kb upstream to the translation start site.

To investigate the divergent expression that has arisen between the two loci, we focused on promoter sequence differences for this study. Alignment of promoter sequences revealed a *FGAM1* stress response element close to the translation start site. Moreover, *FGAM2* promoter constructions showed no GUS expression, suggesting that expression of this locus is much lower or responsive to particular stimuli.

Sequences responsible for feeding site GFP expression were located within an upstream 1.0-kb interval present in both promoters. Observation of enhanced GFP expression in

Fig. 7. Control experiments to assay expression. (A) Test for GUS expression in an untransformed *Arabidopsis* seedling. (B) Examination of nematode entry sites in which the syncytium has not yet been established. A Pr2-2.5 transformant is shown with an arrowhead indicating the nematode head inside root tissues. (C) Test for wound response in a *FGAM1* (Pr1-2.5) transformant. The excised leaf was stained with X-gluc for 30 min. The *FGAM1* deletion constructions, all *FGAM2* constructions and the vector control transformants produced no evident wound response (data not shown).



feeding sites from all constructions, and the considerable sequence homology within the upstream 1.0-kb interval that confers nematode-responsive expression, suggest that nematode-inducible activity was acquired before the gene duplication event.

It is conceivable that nematode responsiveness in the expression of FGAM synthase has facilitated coevolution of the host–nematode interaction. Purine biosynthesis gene expression in the root has already been shown to be inducible by *Rhizobium* (Schnorr et al. 1996). In fact, several examples of reprogrammed plant gene expression have been found in response to nematode infection (Gheysen and Fenoll 2002). Juergensen et al. (2003) demonstrated activated expression of *AtSuc2*, which mediates the transmembrane transfer of sucrose into syncytia that acts as nutrient sinks for the nematode. Down regulation of a novel *Glycine max* ethylene-responsive element-binding protein 1 (GmEREBP1) has also been reported. This protein binds to GCC motifs located within PR gene promoters in *H. glycines*-infected soybean roots during a susceptible interaction (Mazarei et al. 2002) to undermine host defenses. Vercauteren et al. (2002) reports the up-regulation of a pectin acetylesterase gene in feeding sites of root and cyst-knot nematodes. This gene encodes a pectin-degrading enzyme that may be involved in softening and loosening the primary cell wall in nematode-infected plant roots, leading to expansion of the syncytium. These reports reflect the very broad spectrum of genes thought to be redirected in expression by the nematode for feeding site establishment. The feasibility of disrupting gene expression patterns essential to feeding site establishment as a method of plant protection has not been fully assessed.

Sijmons et al. (1991) were first to document in detail the requirements for successful infection of *Arabidopsis* by economically important nematodes. Golinowski et al. (1996, 1997) have undertaken ultrastructural studies on root cellular architecture and have followed the course of development of *H. schachtii* in *Arabidopsis* roots. The nematode developmental life-cycle (~6 weeks) is similar to that of *Heterodera glycines*. Likewise, the sequence of changes in root cell morphology appears to follow a similar course to that in soybean roots. For these reasons we suggest that the observations made in *Arabidopsis* are likely to parallel events in the infected soybean root.

Interestingly, the expression profiles observed in the full length and deletion constructions for the *FGAM1* promoter were similar to the pattern reported for the promoter of gene *pyk20*, isolated from *Arabidopsis thaliana* by a promoter tagging strategy (Puzio et al. 2000). This approach was used to identify genes that were active in nematode feeding sites. The investigators detected expression within the feeding sites as well as floral organs, and a wound response within leaves. Likewise, they reported a region of 963 bp upstream to the first ATG of *pyk20* that was sufficient to direct expression within the nematode feeding site in *Arabidopsis* roots. The lack of expression within feeding sites by vector control constructions (35S::GFP) in our study agrees with previous published data (Urwin et al. 1997; van Poucke et al. 2001).

Opperman et al. (1994) reported a requirement of 300 bp of upstream sequence to the *TobRB7* gene of tobacco for localized expression in *Meloidogyne*-induced giant cells. Moreover, Escobar et al. (1999) identified a sequence 111 bp upstream of the TATA box where nuclear proteins from nematode-induced galls formed DNA protein complexes. These reports indicate that putative nematode responsive domains are generally present in regions of the promoter very close to the transcription initiation sites. It is conceivable that an array of common nematode responsive promoter domains serve as the primary means of coordinating plant gene expression during syncytium establishment.

Based on our observations, it appears that the *FGAM1* locus likely serves housekeeping functions, whereas *FGAM2* may respond to specific environmental stimuli. Yamamoto et al. (2000) reported the cloning of two identical CLAVATA 1 like genes from soybean that show differential expression patterns and suggest that the function of the two genes is slightly different in different organs. Because both FGAM loci are nematode inducible, investigation of the importance of FGAM synthase to syncytium establishment will likely require an inducible gene interference strategy. This experimental approach has not yet been pursued.

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